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Research Article

CD30 expression and Detection of Epstein-Barr Virus-Encoded Small RNA by Real-Time RT-PCR of Classic Hodgkin Lymphoma and Their Correlation with Risk Stratification

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Risk Stratification Hodgkin's Lymphoma

Abstract: More than 40% patient of Hodgkin lymphoma (HL) are chemoresistance and show *early disease relapse that leads to poor prognostic*. Epstein-Barr Virus (EBV) appears to have poor prognostic in HL. The CD30 expression at Hodgkin and Reed-Stenberg cells suggested has associated with advanced disease stage. The International Prognostic Score-7 (IPS) is a seven-parameter of risk stratification index for prognostic and as a consideration for treatment plan. The aim of this journal is to see correlation between EBV infection and CD30 expression with IPS-7 in Classic HL (CHL) patient.

Case-control research design was done to 40 Fixed Formalin Paraffin-Embedded (FFPE) of CHL at the Dr Hasan Sadikin Hospital, Bandung. Cases were 20 patients CHL of high-risk score IPS-7 and controls were 20 patients with low-risk score. Detection of EBV-encoded small RNA-1 (EBER1) by Real-Time RT-PCR and Immunohistochemical staining of CD30 was performed to all samples. All data were analyzed using Chi-Square test with p-value <0.05 of significant level.

EBV was present in 10 cases of CHL (25%). The result of this study shows a different statistically significant of EBV infection (p=0.028) and CD30 expression (p=0.004) between low risk and high-risk IPS-7 HL. CD30 expression (OR= 7.429) may provide stronger factor for risk assessment for the patient with HL, compare to EBER1 detection (OR=6.000).

This study concluded that CD30 expression and EBV Infection correlate with risk assessment IPS-7 in CHL.

Keywords: Hodgkin Lymphoma, EBV, CD30

Introduction

The incidence of Hodgkin Lymphoma *increased* by 17% between 2015 and 2018, according to data from the GLOBOCAN International Agency for Research on Cancer. The mortality-to-incidence ratio was higher in Indonesia (0.56) than in the highest incidence rate of HL in the world, which is Italy (0.1).[1] HL are recognized: nodular lymphocyte predominant HL (NLPHL) and classic HL. In CHL four histological subtypes are distinguished: nodular sclerosis CHL (NSCHL), lymphocyte-rich CHL (LRCHL), mixed cellularity CHL (MCCHL), and lymphocyte-depleted CHL (LDCHL).[2, 3] The etiology of HL is still unknown, but the most suspected is EBV. [4] EBV belongs to the family of herpesviruses [5] and more correlate with CHL than NLPHL, especially MCCHL and LDCHL subtype.[2] EBV detection in HL may be used to risk-stratify patients and derive optimum treatment strategies. [6, 7] EBV life cycle consists of latent and lytic phase, where the latent phase is associated with malignancy. In vitro latent infection of B lymphocytes with EBV is characterized by the expression of latent infection membrane proteins 1 and 2 (LMP1 and LMP2), six EBV nuclear antigens (EBNAs), and two small, nuclear, noncoding RNAs (EBV-encoded RNAs [EBERs]). HL related to EBV latent phase II and will express EBNA1, LMP1, LMP2, EBER and miRNA. [8] EBERs are by far the most abundant viral transcripts expressed in infected cells[9]. Investigation into the presence of EBV nucleic acids in affected tissues in EBV-associated diseases is performed by a variety of different techniques, including *in situ* hybridization (ISH), the polymerase chain reaction (PCR) and immunohistochemistry [6, 7] PCR is the most sensitive method to identify EBER.[10]

CD30 is a member of the tumour necrosis factor receptor superfamily. It is characteristically expressed in certain hematopoietic malignancies, including anaplastic large cell lymphoma and Hodgkin lymphoma, among others. The variable expression of CD30 on both normal and malignant lymphoid cells has focused research efforts on understanding the pathogenesis of CD30 upregulation, its contribution to lymphomagenesis through anti-apoptotic mechanisms, and its effect on cell survival. [11]In Hodgkin's lymphoma, CD30 expression on Reed–Sternberg cells surface is associate with size and invasively of tumor, thus representing a possible indicator of disease aggressively independent by age, race and symptoms [12].

IPS remains prognostic for advanced-stage HL, and each point has different percentage for 5 years freedom for progression and overall survival.[13, 14]

Materials and Methods

Patients

We found 122 cases of HL at Dr. Hasan Sadikin General Hospital between 2014 and 2018. Among them, tissue block and 7 clinical parameter data of IPS were available for 40 cases. All patients were pathologically confirmed to have CHL. The patients were divided into 20 patient of *low risk* (IPS Score 0-2) and 20 patient of *high risk* (IPS Score ≥3). All histological and immunophenotypic data were reviewed by two pathologists (S.S and B.S.H.). The cases of CHL were subtyped according to the World Health Organization 2017, criteria as NSCHL, LRCHL, MCCHL, or LDCHL.

Immunohistochemistry of CD30

Paraffin sections were stained with CD30 antigen using a standard SP immunohistochemistry kit supplied by Biocare Medical (Mouse, Monoclonal, California, USA). CD30 samples were appreciated semi-quantitative as follows: low expression was (+1) distribution 1-25% Hodgkin Reed-Stenberg (HRS) cells positive; and high expression was (+2) distribution 26-75% HRS cells positive, and (+3) 76–100% HRS cells positive, then its divided into low category (+1) and high category (+2 and +3).

Polymerase Chain Reaction (PCR)

Paraffin-Embedded block were cut into 4 × 5 µm thick section. These microdissected tumor tissues were put into 1.5 ml microcentrifuge tubes, then RNA were extracted and purification with *Quick*-RNA™ FFPE Kit (Zymo Research, California, USA). Before EBV specific amplifications, RNA samples were quantified with Qubit 3.0 Fluorometer. Real time Reverse Transcriptase PCR (Real-Time RT-PCR) of 5 ul of Total RNA template samples were used to amplify EBER1 primers reverse and forward. Reverse Transcription was performed at 95°C for 3 minutes, polymerase activation was performed for 40 cycles at 95°C for 5 minutes, cycling conditions are as follows: denaturation 95°C for 10 s, annealing/extension 52°C for 30s, then measure the fluorescence signal. Qualitative analyses of the data were carried out using Light Cycler 480 (Applied Roche, Basel, Switzerland). Reaction concentrations and conditions were adjusted according to the manual instructions of qPCR *SyGreen 1-Step Detect Lo-ROX* (Pcrbio system, Macherey-Nagel, Duren, Jerman) The sequences of the primers are as follows: GAT CCA AAC TTT AGT TTT AG (EBER1 forward), and GCG AAC CGT AAC TCT ATA C (EBER1 reverse).

Result

Patient Characteristic

The clinical characteristics of the 40 patients from each group are summarized in Table 1. The P value show that characteristic patient in Age, gender and subtype are homogenous, except the stadium parameter.

Table 1. Characteristic Patient from each group

Characteristic	IPS		P Value
	Low Risk (<3) N=20	*High Risk (≤3)* N=20	
Age Median (Range)	36.5 (5-65)	40.5 (13-76)	0.517
<45 years	14(70.0%)	12(60.0%)	
Male Gender	13(65.0%)	14(70.0%)	0.507
Subtype			0.927
MCCHL	7(35.0%)	6(30.0%)	
NSCHL	4(20.0%)	5(25.0%)	
LRCHL	7(35.0%)	6(30.0%)	
LDCHL	2(10.0%)	3(15.0%)	
Ann Arbor Stage			**0.035**
I	4(20.0%)	0(0.0%)	
II	8(40.0%)	3(15.0%)	
III	6(30.0%)	9(45.0%)	
IV	2(10.0%)	8(40.0%)	

CD30 expression in CHL tissue

The correlations between CD30 and characteristic variables are summarized in Table 2. sixteen patients of high risk group (80%) displayed high positivity (distribution >25%) for CD30 expression (Fig. 1A, 1B and 1C). There was correlation between CD30 expression and IPS, with P value 0.004 (P value <0.05), with Odd Ratio 7.429 and confidence interval 1.778-31.040. patients with high category of CD30 expression (distribution> 25%) will get High-Risk IPS-7 7,429 times higher compared to patients with low CD30 expression (distribution (≤25%). There was no correlation between CD30 expression and age, gender, subtype and stadium variables.

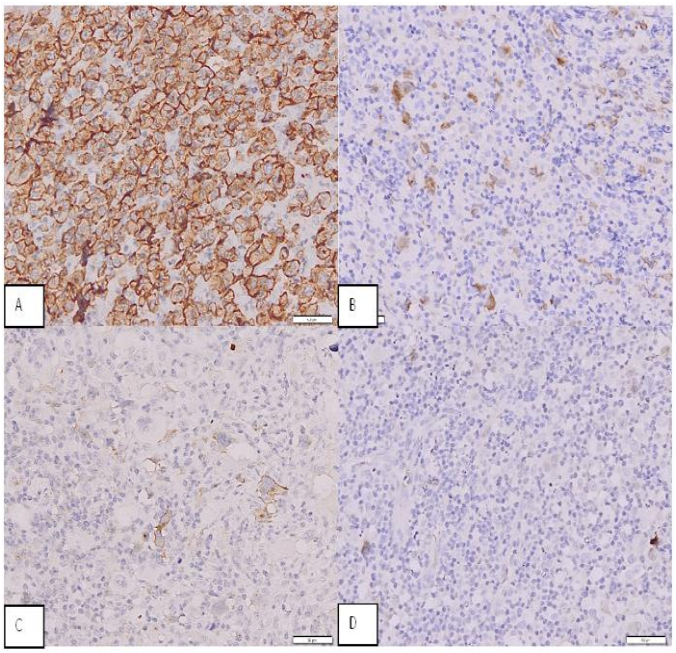

Figure 1. CD30 Expression A. High Category : Distribution >75% (200x) B. and C. High Category: Distribution 26-75% (200x) D. Low Category: Distribution <25% (200x)

Table 2. Correlations between characteristic variables and CD30 expression in all cases

Characteristic	CD30 Expression Low (≤25%) n=17	CD30 Expression High (>25%) n=23	P Value
Age (yr)			0.5714
<45	10	13	
≥45	7	10	
Gender			0.3836
Male	12	14	
Female	5	9	
Subtype			0.490
MCCHL	6	7	
NSCHL	1	7	
LRCHL	7	7	
LDCHL	3	2	
IPS			*0.004
Low risk <3	13	4	
High Risk ≥3	7	16	
Stadium			0.481
I	2	2	
II	7	4	
III	5	10	
IV	3	7	

EBER1 detection in CHL tissue

The correlations between EBER1 detection and characteristic variables are summarized in Table 2. Ten patients are positive for EBER1 detection (25%), 8 patients from high-risk groups and 2 patient from low-risk group. There was correlation between EBER1 detection and IPS, with P value 0.028 (P value <0.05). Odd Ratio value was 6.000 with confidence interval 1.082-33.274. It concluded that patients with EBER1 positive will get High-Risk IPS-7 6.000 times higher compared to patients with EBER1 negative. There was no correlation between EBER1 detection and age, gender, subtype, and stadium variables.

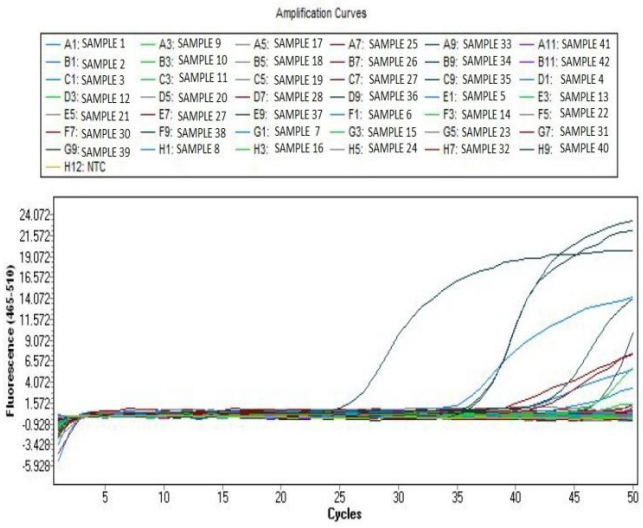

Figure 2. Real-Time RT-PCR showing amplification sigmoid curve in 10 sample. NTC: Negative Control

Table 2. Correlations between characteristic variables and EBER1 detection in all cases

Characteristic	EBER1 Positive n=10	EBER1 Negative n=30	P Value
Age (yr)			0.7175
<45	6	20	
≥45	4	10	
Gender			
Male	9	18	0.1238
Female	1	12	
Subtype			0.764
MCCHL	4	9	
NSCHL	1	7	
LRCHL	4	10	
LDCHL	1	4	
IPS			***0.028**
Low risk <3	2	18	
High Risk ≥3	8	12	
Stadium			0.585
I	2	2	
II	2	9	
III	3	12	
IV	3	7	

After multivariate testing, it was found that there was no correlation between CD30 expression and EBER1 detection simultaneously in predicting IPS7 risk stratification, and it was found that based on the Odd ratio value, CD30 expression (OR: 7,429) was more associated with IPS7 risk stratification, than EBV infection (OR: 6,000). This is thought due to the presence of different pathways in the pathogenesis of HL.

Discussion

EBER1 (EBV *encoded small RNA)* is EBV gen product that expressed in latency II phase and correlated with malignancy, especially HL and nasopharyngeal carcinoma. EBV infection associated with a high risk of IPS-7 stratification is supported by research conducted by Hohaus et al., The results obtained by finding EBVDNA in plasma HL patients, are indicators of disease activity and biological characteristics, which are associated with poor prognosis.[15] The study by Kanarky et al., concluded that pretreatment plasma EBV-DNA is highly concordant with EBER-ISH in classical HL, confirming many previous reports. Pretreatment plasma EBV-DNA appears to have prognostic value and yield information beyond the IPS or its components and had inferior failure-free survival compared with plasma EBV(-) patients. [16] Grywalska *et al*. was recognized that relapsing EBV-related HL had a worse prognosis than non-EBV-related HL. [17] Myriam *et al*. revealed a negative impact of EBV on the survival of patients

with limited Ann Arbor stages (I and II), with nodular sclerosis HL subtype, and in patients without bulky mediastinal tumor. These findings suggest that the EBV affects the outcome of particular subgroups of HL, especially those with early-stage of the disease. [18] Koh *et al*. reported that EBV-positive HL differs from EBV-negative HL with respect to several clinicopathologic variables, including gender, age, stage, IPS status, and the presence of B symptoms. They also show that EBV-positive HL patients aged 25 years or older have a poorer prognosis, particularly if HL is advanced. [19] In most studies conducted, it has been found that EBV infection has a relationship with survival rates in older HL patients, concluding that EBV-related HL patient have a lower survival rate, compared to non EBV-related HL.[20, 21] Keegan's *et al.* concluded that In HL, EBV tumor cell presence is associated with better survival in young patients and poorer survival in older patients with NS, independent of other factors. Evidence that EBV is a meaningful prognostic marker may have therapeutic relevance.[20]

The CD30 molecule was subsequently cloned and characterized as a 120 kD transmembrane glycoprotein receptor belonging to the tumor necrosis factor receptor (TNFR) superfamily, with intracellular, trans-membrane and extracellular domains[22]. Several viruses have been implicated in inducing CD30 expression, including Epstein-Barr virus and human T-cell[23].

In our study CD30 expression is significantly correlate with IPS-7 risk stratification. This is consistent with research conducted by Flangea *et al*, concluded that the increasing of CD30 expression is associated with advanced disease stage.[12]

Su *et al.* concluded that H-RS cells are able to inhibit the proliferation and activation of T cells through CD30-related interaction. The outcome of CD30-related interaction is an ineffective antitumor immunity, which is clearly in favor of the growth and survival of the tumor cells [24]. Van der weyden *et al* found that CD30 mediates its effects through a number of diverse signaling pathways, which in concert confer a survival benefit to the cells on which CD30 is upregulated. Stimulation of the CD30 molecule results in trimerization and signal mediation through tumor necrosis factor receptor-associated proteins (TRAF), in particular TRAF2, but also TRAF1 and TRAF5, to stimulate the nuclear factor-kappa B (NFkB) pathway. In addition to this, CD30 ligation also signals through the mitogen-activated protein kinase (MAPK) pathways, including ERK1 and ERK2, which have diverse anti-apoptotic and pro-survival benefits in the neoplastic cell [11]

EBV infection found in 40% of HL cases, through LMP1 that responsible for the constitutive activation of classic and alternative NF-κB pathways. Classical pathway will involve CD30, CD40, and RANK, while alternative pathways will involve CD40 and BCMA. In the classic NF-κB signaling pathway, stimulation of various receptors, which complex with TNF receptor–associated factors (TRAFs) and the receptor interacting protein (RIP), leads to activation of the IKK complex, targeting the NF-κB inhibitors IκBα and IκB for ubiquitination and proteasomal degradation. As a consequence, the NF-κB transcription factors translocate into the nucleus, where they activate multiple genes. In the alternative NF-κB pathway, activation of receptors such as CD40 and TACI causes stimulation of the kinase NIK, which then activates an IKKα complex. NIK activity is negatively regulated by TRAF3. Activated IKKα processes p100 to p52, which translocates as p52/RELB heterodimers into the nucleus. CD30 and LMP1 signaling can activate both the classic and the alternative pathway of NF-κB and directly contributes to HRS cell survival [25]. Numerous genetic lesions and signaling through the EBV-encoded latent membrane protein 1 in EBV-positive cases of HL play important roles in the deregulated NF-κB activity. The JAK/STAT pathway is the main signaling pathway for cytokines. In HRS cells, STAT3, -5, and -6 are constitutively active. In addition to activation of cytokine receptors, such as the IL-13 receptor and the IL-21 receptor, activation of this pathway is mediated by genomic gains or translocations of the JAK2 gene (30% cases) and frequent inactivating mutations of the SOCS1 gene (40% cases) [3, 26, 27].

Liu *et al.* found that EBER1 suppressed $p21^{cip1/waf1}$ in HL cell lines through down-regulation of p53, EGR1, and STAT1, and EBER1+ HL cell lines were more resistant to apoptosis induced by histone deacetylase inhibitors or proteasome inhibitors. Because these drugs were known to act by increasing $p21^{cip1/waf1}$, the anti-apoptotic activity of EBER1 was probably through the suppression of $p21^{cip1/waf1}$. Clinically, EBV-related HL had weaker expression of $p21^{cip1/waf1}$ and a worse prognosis, which also supported a critical role of EBER1 in the rescue of Reed-Sternberg cells from apoptosis and in the clinical behaviors of HLs [28]. The frequency of genetic lesions or viral infections that affect the activity of the NF-κB pathway and the STAT pathway in HRS cells is varied. [3, 27] CD30 and EBV infection through LMP1 will be in one pathway, which is classic NF-κB pathway. CD30 with EBER1 is also in one pathway on MAPK activation. However, there are other pathways in the pathogenesis of HL, where EBER1 will activate the JAK / STAT pathway, that the activation of this pathway does not involve CD30, and there is also a pathway where CD30 runs alone by inhibiting T cell proliferation, thereby increasing HL progression. The JAK / STAT pathway activated by EBER1 or the T cell proliferation inhibition pathway by CD30 is thought to be a widely used pathway in the pathogenesis of HL in this study sample, resulting in EBER1 not running simultaneously with CD30 to be associated with IPS-7 risk stratification.[29, 30]

In summary, CD30 expression has utility not only for differential diagnostic, but also has prognostic value. EBER1 positivity was correlated with high-risk score IPS-7 among patients with CHL. Our results suggest that CD30 expression and EBER1 Detection are significantly associated with IPS-7, but not simultaneously.

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